Reprint from

Behring Institute
Research Communications

Behring Institute Mitteilungen

No. 51 · September · 1972

ALG

Therapy and Standardization Workshop

Bad Soden (Ts)
April, 23rd – 25th, 1972

Published by
Behringwerke AG, Marburg
Edited by
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Behringwerke AG

D 355 – Marburg / Lahn
Germany

Summary Statement for Session (1) on In Vivo and In Vitro Testing of ALG

E. M. LANCE

In vivo testing

From the data presented at this meeting it appears that most species of Macaques monkeys are suitable for demonstrating the immunosuppressive potency of anti human lymphocyte sera. Some of the subspecies appear to be more sensitive, such as the rhesus and speciosa, while Patas monkeys appear to be refractory. Some evidence suggests that baboons and cynomolgus monkeys are slightly less sensitive when used for this purpose.

The evidence that the results of assays in monkeys and immunosuppressive potency in man are related remains at this time circumstantial and there is no conclusive proof that these assays are predictive for man. Even if they are predictive we do not yet know whether there will be direct proportionality between potency in monkeys and potency in man.

There are a number of problems with the monkey, or surrogate assay as it is currently being performed. These include the lack of standardization amongst the various testing facilities particularly with respect to the species of monkey used, the number of animals employed in screening for any one antiserum, and the treatment schedules. These differences create difficulties in attempting to make cross comparisons from laboratory to laboratory.

Apart from revealing potency, in vivo testing in monkeys can be used to detect toxicity. Acute systemic toxicity, hemolysis and thrombopenia as well as nephrotoxicity may be revealed and are probably predictive of noxious antibody classes. There are in addition some adverse reactions which have been observed in subhuman primates the mechanism of which is poorly understood. It is clear that intravenous administration is sometimes lethal and this may be in part explained by the phenomenon of reversed anaphylaxis. Therefore, screening in subhuman primates for toxicity is a valuable safeguard prior to clinical application.

In the future it would be highly desirable for the various facilities which are conducting this sort of test on a large scale to standardize their techniques. This standardization should apply to all the variables such as species, dosage

schedule, the number of animals used for each antiserum, as well as the number of skin grafts applied to each monkey. In this way only will it be possible to define antisera in units of potency which will be comparable throughout the world. This in turn will make the interpretation of clinical trials more meaningful.

In vitro assays

All of the various test systems which were described at this meeting gave a good correlation within a species. That is, a high titer in the various tests usually was predictive of skin allograft prolongation in the appropriate experimental animal. Again these tests when used to titer anti human ALG showed a statistically significant correlation with the results of the surrogate host assay. However, some tests which were not discussed at this meeting such as opsonisation have also been reported to show a significant correlation. Moreover, there appears to be a good correlation between the titers obtained in one in vitro assay system with those obtained in another.

A difficulty that arises is that the product which is submitted to testing in the various laboratories is quite different. Some people use whole antiserum, others only IgG. The type and amount of absorption prior to testing varies from laboratory to laboratory and these factors create problems when one attempts cross-comparison from test to test. We believe it is highly desirable for in vitro testing to be confined to the product which is likely to be used in man, i. e. highly purified IgG which has been absorbed to remove noxious antibody classes.

It is not possible at this stage to select any single test that possesses virtues which exceed that of any other. This will only become possible when reference sera have been tested in man and crosscorrelated with the various in vitro assays. At the moment it is probably sound policy to employ a battery of these tests. There may be safety in numbers and those assays which have at least the advantage of simplicity so that they can be applied by most laboratories appear to be rosette inhibition, indirect immunofluorescence, microcomplement fixation, indirect passive hemagglutination, and the cytotoxicity assay.

Summary Statement for Session (2) Regarding Production and Purification

H. BALNER

Species

It has been shown to be possible to raise effective ALG in numerous mammalian species: The *horse* is still most widely used but in view of the possibility of anaphylaxis, the availability of ALS raised in a not closely related species such as the *rabbit* is recommended.

However, rabbit ALG should be used carefully in bone marrow transplantation since it may be damaging to hemopoietic stem cells. Furthermore, *goat* anti-human globulins were shown to be effective and remarkably devoid of toxic side effects.

Antigen

There is still a rather wide choice of eligible sources of human lymphoid antigen. Some antigen sources such as spleen and blood lymphocytes should be excluded mainly because it is difficult to remove contaminants leading to toxic, undesirable antibodies.

Thoracic duct cells seem to be an excellent antigen but not easy to obtain.

Thymocytes are widely and successfully employed but stromal elements should be carefully removed to avoid formation of GBM-reactive antibodies.

Cultured lymphoblasts have the advantage of constant quality and availability within a particular line. However, the characteristics (and content of relevant antigens) may differ from line to line and *viral* contamination may be a hazard.

In contrast to impressions gained at previous ALS conferences the efficacy of antilymphoblast sera seems less consistent than that of antithymocyte sera although methods to evaluate efficacy are obviously imperfect.

As yet, insufficient data are available regarding optimal methods of storing cellular antigens.

Membrane preparations have been used successfully. They offer the advantage of uniformity, easy storage and, most importantly, the possibility to *select the relevant* and eliminate irrelevant antigens. However, further standardization is necessary to extract the optimal membrane fractions from human lymphoid cells.

Production

There are numerous acceptable immunization methods. While the i. v. "two-pulse" method is still used to raise ALG in small animals there is an increasing use of various adjuvant methods especially in larger animals.

The importance of producing *very large pools of uniform standardized* material is once again emphasized. To achieve this aim, highly rationalized production methods are essential; selection and pooling of batches requires thorough and repeated in vitro and in vivo testing to ensure i. s. potency and lack of toxicity.

Several *purification methods* can be used to extract the active globulins from crude serum. Agreement has been reached that the i. s. principle resides in the IgG fraction. For *horse* sera a further restriction to certain subclasses can be made (in fact the electrophoretically slow γ_2 fraction is usually optimal).

The problem of long-term storage of ALG urgently needs further investigation.

Summary Statement of Session (3) and (4): Kidney Transplantation

A. P. MONACO:

The following represents the summary prepared by *Dr. Pichlmayr* and I on the clinical transplantation reports of ALG-therapy.

13 series of clinical renal transplantation were presented utilizing ALG as adjunctive immunosuppressive therapy in association with conventional therapy of Imuran, prednisone and, in one instance, cyclophosphamide.

The potential effectiveness of ALG was examined in 8 areas:

1. Overall Survival Rates

There was no clearcut demonstration that ALG improved one or two year overall-survival of kidney and/or patients. At least 3 concurrent series with non-ALG controls failed to show an increase in overall survival. On the other hand, one and possibly two retrospective series clearly showed a definite increase in one year patient and kidney survival.

2. Quality of Function of Surviving Grafts

The point seems to be reasonably well established by several series, particularly the concurrent series, that surviving ALG-patients show better renal function at one year (as determined by creatinine clearance and serum creatinine levels) over surviving non-ALG-treated patients.

3. Levels of Steroids Required for Kidney Survival

A very likely salutary effect of ALG is a decrease in the steroid requirement for maintenance of renal function in surviving patients. Furthermore, at least one series demonstrated a concomitant reduction of complications associated with steroids in surviving patients treated with ALG.

4. Incidence and Severity of the Rejection Reactions

The majority of the ALG-treated series both with concurrent and retrospective controls demonstrated a definite and highly significant fall in the incidence of post-transplantation rejection reactions. Thus, there were in the order of 1,5 rejection reactions per patient per year in non-ALG recipients versus 0,5 rejections reactions per patient per year in ALG recipients. Furthermore, all agree that the severity of the rejection reactions, when they did occur, was much less as measured by ease of reversal, the time required to return creatinine to normal levels, and the necessity for return to dialysis. Finally, many speakers on this point suggested that the overall general management of the patients was much easier in the ALG groups in terms of length of hospital stay etc.

5. Use of ALG in the Treatment of Rejection Reactions

One series which failed to demonstrate an overall salutary effect of ALG to improve survival rates of kidney and/or patients noted an almost unusual effectiveness of ALG in treatment of rejection reactions. They were so impressed that they regarded use of ALG to be primarily for rejection treatment. Another series suggested a modest good effect of ALG in the rejection treatment but emphasized that best effectiveness was achieved when steroids were raised concurrently. However, the majority of series failed to state, imply, or support the effectiveness in rejection reactions.

6. Effectiveness in Sensitized Patients and Second-Transplant Patients

Several series reported results which suggested that the use of ALG in presensitized patients, i. e. crossmatch negative donor-recipient pairs, but recipients containing antibodies to a normal lymphocyte panel, failed to improve results in these potentially more difficult patients. On the other hand, one large controlled concurrent series considered the results of presensitized and second-transplant recipients better when given ALG and they proposed that this group of patients comprised an area where ALG was definitely indicated for therapy.

7. Dose Effect

With regard to differences of doses, at least two large series were able to show that higher doses of ALG on a mg/kg basis were preferable to lower doses. On the other hand when these two higher dose series are analyzed their overall survival rates were no better than any other series using somewhat lower doses. This apparently reflects differences in ALG potency.

8. Substitution for Other Drugs

No series reported regular substitution of ALG for one or more of the conventional immunosuppressive drugs. Several instances were cited, however, where one conventional drug could be removed, because of various emergencies and kidney function maintained in ALG-treated patients.

The potential harmfulness of ALG therapy was evaluated in five problem areas:

1. Survival Rates

Fortunately, no series reported a decrease in either patient or kidney survival in ALG-treated patients.

2. Infections

No series reported a clearcut increase in infections in ALG-patients although several implied that this might be true.

On the other hand it must be emphasized that clearcut reductions in rejection reactions without obvious increase in one and two years survival suggested some negative effect might be operative and this might be in the area of serious or fatal infections.

3. Direct Damage to Kidney Transplants

Several instances were cited where kidneys were damaged by anti-GBM-antibody contained in ALG. The majority of series, however, failed to consider this a significant danger and failed to show evidence of significant anti-kidney effect from ALG.

4. Tumors

No increased incidence in tumors were cited by any series.

5. Economic Problems and Cost

The cost of ALG-production in certain instances was definitely cited as a significant deterrent for its routine usage relative to the demonstrable benefit which could be attributed to it.

Finally, in terms of future prospects, in the panel discussion, all agreed that ALG seemed to be beneficial particular in terms of rejection reactions. There seemed to be an overall impression, however, which was explained and somewhat disappointing, that overall-one-year survival results had not been improved. Finally, I think it was the majority of opinions that more concurrent-controlled studies should be done.

Summary of Session (8): Tumor Progression Risk

D. W. van BEKKUM

1. There is overwhelming evidence both in animals and man that tumor incidence increased under continued immunosuppressive treatment.

2. There is no evidence that this is a specific property of ALG. More experimental work is required to exclude the possibility that the administration of foreign proteins might predispose for certain tumor types such as lymphosarcomas. Careful retrospective analysis of human cases, including the study of their complete immunosuppressive history, remains necessary. In experimental animals in most cases ALS was given *alone*. It is probably more relevant to include in future studies combination regimens, as are employed in clinical work. Controls for non-specific factors, that is, groups not receiving oncogenic stimulation but only immunosuppressive agents, should more often be included. Finally, the possible causative role of the underlying diseases, genetical constitution etc., have to be kept in mind and for this the group of patients that received kidneys from identical twins and have not received immunosuppressive therapy are particularly suited as "controls". Maybe this group which is not so closely subjected to follow-up should be given special attention.

3. More experimental information is needed on the intrinsic oncogenic properties of conventional immunosuppressive agents, such as Imuran, and even the steroids which, among others, kill lymphocytes, should be more carefully studied in this respect.

4. As to the theoretical aspects of the mechanisms underlying carcinogenesis, these are changing rapidly. The concepts concerning immunological surveillance are being modified accordingly. If we want to establish the significance of the immune system by using long-term immunosuppressive regimens, we should request that the animals are being monitored for their degree of immunosuppression, preferably during the whole study. This is now being recognized by most workers, but further perfectioning of suitable techniques is necessary in order to make this possible.

   In studies of surveillance mechanisms, more careful distinction should be attempted between increased susceptibility to infection by viruses – oncogenic or other – and decreased defense against tumor specific antigens.

5. There is conflicting clinical information about the influence of continued immunosuppression on tumor progression and on metastases-formation. Here the experimental evidence clearly indicates that one determinative factor is the antigenicity of the tumor, and all we can recommend therefore, is that the fullest information on this property be collected on all cancer cases. This group of patients constitutes an extremely valuable reservoir of information on human cancer biology and it is strongly recommended to Cancer Agencies to provide funds to transplantation biologists and immunologists to exploit this reservoir of information to the fullest.

Summary of Session (9): Tolerance and Enhancement in Relation to Use of ALG

P. B. MEDAWAR

There is solid and voluminous evidence from small laboratory animals that ALG can be used to promote tolerance when used in combination with antigen. But more important, there is also convincing evidence that ALG can be used to promote tolerance in those larger experimental animals that are widely accepted as faithful models of human reactivity, I mean dogs and subhuman primates. On the controversial question of relationship with tolerance and enhancement various views which question, various degrees of conviction, but it can't be said that any final conclusion was come to. I think it was generally agreed that the inception of tolerance remains an ambition for all organ transplantation with all immunosuppressive agents but no one propounded a method applicable to man by which this ambition could be achieved. In that respect this particular session did not come to a firm single conclusion.

Summary of the Bad Soden ALG-Workshop

T. E. STARZL

It was disconcerting to realize from an earlier meeting in San Diego, California, last December, the extent to which many really central practical questions about ALG had yet to be answered. Some of these questions have been at least partially clarified at this present conference.

Certainly, anti-human ALG is or can be immunosuppressive. In proper doses the expression of preexisting delayed hypersensitivity is prevented and skin graft survival is prolonged. ALG has apparently been a key factor in making possible successful bone marrow transplantation between HLA non-identical donors and recipients as reported by *Schwarzenberg*. It was probably also a major factor in another break-through achievement – that of skin homotransplantation by *Diethelm* in a burned child who would almost certainly have died otherwise.

I doubt if there is an appropriately informed responsible scientist in the world who does not concede that ALG is a potent immunosuppressive agent in humans. But that is not the question which perplexes clinicians interested in renal transplantation. Rather, the issue is whether or not ALG fills some unique role that cannot be equally well met by the clever manipulation of other agents such as steroids, azathioprine and cyclophosphamide. Opinions about this were varied. Testimony about the value of ALG in renal transplantation was given from 13 centers. Eight positive votes were cast, but 4 clinicians did not believe that their results were improved, and one European surgeon thought they were actually worsened.

The only controlled study in cadaveric renal transplantation that has yet been performed was brought up to date by *Dr. Ross Sheil* of Australia. His patients, who received a 2 months course of goat ALG in addition to maintenance therapy with azathioprine and prednisone fared better than those who did not get globulin. However, the differences were not overwhelming. Consequently, this small series, important and wisely planned as it was, must now have reinforcement. Although we ourselves have resisted the compilation of a control series without ALG because of our conviction of the value of the globulin, I strongly support anyone who wishes to carry out such a controlled study and we will have to give consideration to this possibility ourselves.

One reason why the question of indispensability of ALG must be settled is the tremendous investment of personal and material resources that have been required to make ALG available for human use. We heard examples in which the cost of ALG accounted for half the financial investment to treat a renal recipient. In addition, I am amazed at the amount of talent that is required to ensure a supply. These efforts and expenses will be worthwhile only if tangible and substantial benefits are demonstrable.

If such tangible benefits are not readily discernible in well controlled studies this sidecorner of transplantation is going to undergo acute atrophy. In addition to the nuisance of procuring it there are potential dangers with the administration of ALG. Anaphylaxis, which has led to several deaths, is the most terrifying side effect, but there are others including injection site pain, thrombocytopenia and injury to the homograft itself to mention only three. The Minnesota group has reported serious thrombotic complications with some high titer ALG which was given i. v. in their center and elsewhere. Apparently these thrombotic calamities were caused by crossreactions of the anti-white cell antibodies with recipient platelets.

Of course, I do not know what the results of future controlled clinical trials will be but if as I suspect, they prove to be positive, it will be an enormous stimulus for companies like

Behringwerke. Then the consumer cost would fall. In the long run the greatest market for commercial ALG might well be for patients with autoimmune disorders rather than for transplant recipients. The possibilities were discussed by Drs. *Brendel*, *Pirofsky*, and *Traeger*, who all emphasized the difficulty of evaluating the results in these disorders that so characteristically undergo spontaneous remission and exacerbations. Sympathetic ophthalmia, from the reports we heard, may be an unusually specific indication for ALG treatment. In autoimmune disorders, as in transplantation, ALG, if given without other potent immunosuppressants may be excessively dangerous.

Even if highly successful clinical transplantation trials are carried out, there will remain very major problems of standardization. It is not my responsibility to discuss these matters today, but I will mention four exceptionally sensitive points that must be clarified: first, the best animal in which to raise ALS, second, the most effective immunization schedule to be used, third, the correct antigen, and finally the *in vitro* and *in vivo* techniques for evaluating the effectiveness of the product.

Personally, I doubt that the choice of animal is a crucial factor, a view supported by the studies of *Simpson* and of *Barnes*. The schedule of immunization is probably also not critical except that if the course is a short and standard one according to the *Monaco-Medawar* principle the ultimate product is apt to be relatively the same from animal to animal. In horses this was shown by *Groth's* data from 5 animals submitted to 3-pulse immunization with large numbers of lymphoblasts.

The third question about the best antigen source is still open for discussion. The thymocyte has a number of advocates including *Barnes*, *Kayhoe*, *Sheil*, and *Pirofsky*. From the viewpoint of convenience and purity a contender is the cultured lymphoblast which as far as we know represents a pure B-cell population. There have been disquieting reports in the surrogate monkey system, from *Kayhoe's* laboratory, of a lack of a strong immunosuppressive effect in all anti-lymphoblast sera studied so far by these workers. In contrast *Seiler* and *Balner* have seen many strong anti-lymphoblast preparations, and of course the classical Minnesota dose-response curves in humans were derived with this kind of ALG. Consequently it is my guess that the lymphoblast will be the antigen of the future. Alternatively *Lance* made a strong case for stored lymphocyte cell membranes.

Concerning the fourth point, there has been a gradual acceptance at least of at least 4 *in vitro* tests. Even a year ago there were flat denials that the leukoagglutinin and lymphocytotoxicity tests had any correlation with immunosuppressive effect although it was commonly conceded that correlations were good with the *Rosette* inhibition test of *Bach*. Yet on Sunday of this meeting, Dr. *Kayhoe* told us that with rabbit ALG the cytotoxicity titers and potency in the monkey skin test system had an almost perfect correlation. In beautiful studies with the horse *Seiler* has as well as *Johannsen* shown that 4 current titration methods all yielded about the same answer – cytotoxicity, lymphoagglutination, rosette inhibition and micro-complement fixation – and that the heights of these titers were a relatively direct measure of the immunosuppressive quality as cross checked in the surrogate monkey model. *Seiler* pointed out that methodologic artifacts in measurement of anti-white cell titers may have accounted for discrepancies in the past. It was interesting that additional more or less new titration methods were described using indirect agglutination techniques both by *Monaco* and *Revillard*, and using an indirect immunofluorescence technique by *Edwards*.

It may be superfluous to engage in much debate about the dose and administration schedules of such a poorly standardized agent as ALG. Nevertheless, it is important to attempt this by those who are or are planning to give ALG now. We have always believed that ALG for human use should have a minimum leukoagglutination and cytotoxicity titer of 1:8000. We give adult patients ALG of this potency in volumes of 4–5 ml per injection intramuscularly. Since the protein content of our material

is 5 mg % the dose per injection is approximately 4 to 5 mg/kg. It should be noted that this kind of dose in the *Simmons* dose-response curve caused an easily detectable prolongation of human skin graft survival. I am very much afraid that if these doses are further reduced either by using poor titer material or by decreasing the volume of injectate one may easily enter into homeopathic range. If alternating case studies of renal transplantation are carried out I hope that ALG will not be discredited by making this mistake of underdosage. It may be noted that *Sheil* takes strong account of his rosette inhibition titer in his dose scheduling. *Traeger* has also emphasized the need for adequate dosage.

The *duration* of ALG injections used together with a cytotoxic drug plus prednisone has ranged in clinical trial from 2 weeks to several months. How can we logically expect a lasting benefit in a human being from such a short therapeutic course. Sir *Peter Medawar's* session on tolerance, enhancement, and the popular notion of a combination of these factors promoting graft acceptance have provided us with some speculative answers. In a crude way, it is appreciated that heavy early immunosuppression may promote graft acceptance by these means or by others not understood and that consequently the benefit of short but intense therapy with ALG may far outlast the actual time of treatment. There were reports from *Monaco, Myburgh, Lance* and *Wilson* about biologic "adjuvants" which might be used to facilitate graft acceptance at this critical early postoperative time. The techniques included the administration of tolerogenic antigens in the form of donor bone marrow or lymphoid cells or non-cellular extracts of lymphoid tissues along with or shortly after the transplantation of kidneys, livers, or whole limbs. Additively or alternatively, the evaluation of enhancing antibodies was described.

In closing a very brief comment is in order about the development of malignancies in patients subjected to acute or chronic immunosuppression. There is no question that *de novo* malignancies occur in human recipients of renal homografts at an abnormally high incidence, although this complication by no means vitiates the value of the procedure. There is no time to dwell on this subject, beyond saying that the neoplasias are not associated especially with ALG, and that for reasons discussed yesterday by *Medawar* and his panel, having to do with the ability to reduce the level of chronic immunosuppression, the opposite may actually prove to be true.

Final Remarks

H. G. SCHWICK

Ladies and Gentlemen, our meeting is coming to the end. I hope that this workshop has been useful to everyone so that the long journeys were worthwhile. I think nobody was so optimistic to assume that during this meeting all problems concerning ALG would be solved. But may be at least we approached to the solution. In any case the discussions have led to an exchange in informations so far available and this is always the basis for progress in science.

I like to thank all the chairmen who have led us through this extensive program. I like especially to thank my coworker Dr. *Seiler* who has done the whole organization work.

I wish you a pleasant trip home.